Use of Sulfuric Acid in the Detection And Estimation of Steroidal Sapogenins

HENRY A. WALENS, ARTHUR TURNER, JR., and MONROE E. WALL
Eastern Regional Research Laboratory, Philadelphia 18, Pa.

A rapid method for the identification and estimation of steroidal sapogenins was required. It was found that steroidal sapogenins dissolved in 94% sulfuric acid have characteristic ultraviolet absorption spectra in the region 220 to 400 mμ, which can be used for the detection and estimation of these substances. Optimum reaction conditions are the use of 0.1 to 5.0 mg. of sapogenin dissolved in 10 ml. of 94% sulfuric acid and warmed at 40° C. for 16 hours. Under these conditions, the determinations are reproducible and follow Beer's law. Constituents of binary mixtures can be spectrophotometrically determined, using appropriate equations based on previously determined absorptivities. More complex mixtures can be determined after preliminary separation by means of chromatography. A qualitative scheme is suggested for the identification of the 13 most commonly occurring steroidal sapogenins, using chromatographic behavior, spectra of the sulfuric acid chromogens, and melting points. The method can be applied only to purified sapogenins, not to crude mixtures.

DURING the course of a study on the partition coefficients of steroidal sapogenins, the need for a method for determining relatively small quantities of these compounds became apparent. The method finally adopted was based on the studies of Diaz, Zaffaroni, Rosenkranz, and Djerassi (*1*). They showed that steroidal sapogenins, on treatment with concentrated sulfuric acid, give characteristic chromogens.

It was found necessary to make a thorough study of this reaction, and it has been observed that under suitable conditions the reaction can be used both for identification and for estimation of the various sapogenins.

A qualitative scheme has thus been developed for the identification of the 13 most commonly occurring steroidal sapogenins, using chromatographic behavior, spectra of the sulfuric acid chromogens, and melting points.

PROCEDURE

General. The sample, preferably 5.0 mg. (although quantities as low as 0.1 mg. can be used) is weighed into a 10-ml. volumetric flask. Alternatively, a sample in this weight range is dissolved in chloroform, and transferred to a 10-ml. volumetric flask, and the solvent carefully evaporated to dryness. Sulfuric acid, 94% by volume, is added to the 10-ml. mark. The flask is then immersed for 16 hours in a water bath maintained at 40° C. The flask and contents are then cooled to room temperature and the contents diluted, if necessary, to volume with 94% sulfuric acid.

For the qualitative determination of the sapogenins, the ultraviolet spectrum is obtained over the range 220 to 600 mμ using a Beckman DU spectrophotometer or, preferably, a Cary recording spectrophotometer, with 1.0-cm. matched quartz cells, using 94% sulfuric acid as the blank.

Four spectral curves, as determined by means of a Cary recording spectrophotometer, are shown in Figure 1. By comparison of the spectrum obtained with Figure 2 and Table I, most sapogenins can be easily identified. Confirmatory methods have been previously described (*2*).

Single-Component Samples. For estimation of the quantity of sapogenin present, the absorbance of the sample is measured at 250 mμ. The concentration of the solution can then be calculated, using the absorptivities listed in Table II, and the amount of sapogenin present can be determined.

Table I. Wave Length Positions and Intensities of Absorption Maxima of Sulfuric Acid Chromogens of Steroidal Sapogenins

Sapogenin	Absorption Maxima, Mμ	Log ϵ^a at Corresponding Maxima
Chlorogenin	270, 330, 415	3.96, 4.00, 3.74
Diosgenin	271, 415, 514	3.99, 4.06, 3.64
Desoxydiosgenin	271, 312	4.00, 3.93
Gitogenin	272, 308	4.08, 4.11
Hecogenin	276, 350, 396	4.06, 4.10, 4.24
Desoxyhecogenin	268, 348, 394	3.97, 3.98, 3.61
Hecogenone	269, 347	3.95, 3.89
Kammogenin	233, 272, 349	4.11, 4.02, 3.89
Kryptogenin	280, 383	3.77, 4.06
Manogenin	276, 348, 400, 468	4.00, 4.04, 3.69, 3.44
Markogenin	270, 308	4.00, 3.90
Rockogenin	273, 379	3.85, 4.07
Samogenin	270, 308	4.01, 3.91
Sarsasapogenin	271, 310	3.98, 3.85
Desoxysarsasapogenin	272, 308	3.97, 3.92
Sarsasapogenone	267, 310	4.01, 3.76
Smilagenin	272, 312	3.98, 3.90
Desoxysmilagenin	273, 308	4.00, 3.94
Smilagenone	268, 310	4.06, 3.86
Tigogenin	270, 312	3.94, 3.88
Desoxytigogenin	274, 296	3.99, 4.03
Yuccagenin	240, 268, 405	4.11, 4.09, 3.83
Cholesterol	308, 416	3.95, 3.58

[a] Logarithm of molecular absorptivity.

Table II. Absorptivities of Steroidal Sapogenins at 250 and 350 Mμ[a]

Sapogenin	Absorption Coefficients 250 mμ	350 mμ
Chlorogenin	16.0	18.4
Diosgenin	18.4	14.6
Gitogenin	18.1	8.8
Hecogenin	16.3	29.6
Kammogenin	18.3	17.5
Kryptogenin	9.9	12.3
Manogenin	15.3	23.3
Markogenin	16.6	10.9
Rockogenin	17.9	22.4
Sammogenin	17.2	12.0
Sarsasapogenin	15.3	13.9
Smilagenin	16.4	13.9
Tigogenin	15.7	13.9
Yuccagenin	28.0	12.4

[a] Absorptivity is defined as $a = A/bc$ where A is the absorbance of a solution of thickness b (centimeters) and c grams per liter compared with an equal thickness of solvent.

Binary Mixtures. The procedure for the qualitative identification of binary mixtures is essentially the same as for single-component samples. The estimation of the sapogenin content requires the measurement of the absorbance of the solution at 250 and 350 mμ. The concentration of each component can then be calculated by the use of simultaneous equations, using the absorptivities presented in Table II.

Multicomponent Mixtures. A multicomponent mixture must first be separated into fractions, each fraction being either a single component or a binary mixture. This separation can best be accomplished by means of adsorption chromatography using −80-mesh activated alumina or Florisil (Floridin Co., Warren, Pa.) as the adsorbing agent. Both are heated to 150° C. before use.

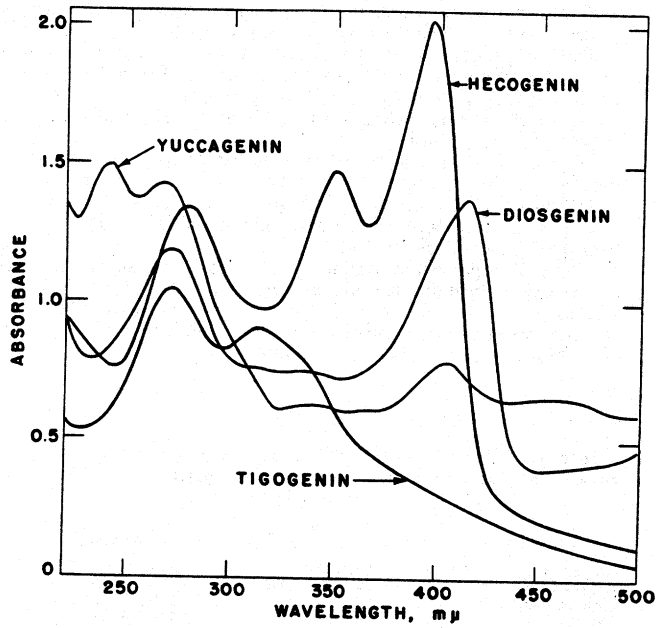


Figure 1. Absorption Spectra of Hecogenin, Tigogenin Diosgenin, and Yuccagenin

Cary recording spectrophotometer

The amount of adsorbing agent used in the chromatography can be greatly varied, depending upon the size of the sample and the size of the columns available. With a relatively small sample (1 gram or less), 50 to 100 times as much adsorbent as sapogenin can be used. This ratio can be lowered to 10 times as much adsorbent as sapogenin with larger samples, but should not be lowered to less than 10 to 1. The column should have a relatively small ratio of cross section to length, and the rate of flow should be low. The elution pattern is essentially the same for both of the adsorbing agents. Alumina is the more active adsorbent, but Florisil is preferable as a resin remover.

In the procedure using Florisil, the sample is dissolved in as small a volume of hot benzene as possible, and the solution is cooled to room temperature. Any undissolved or reprecipitated

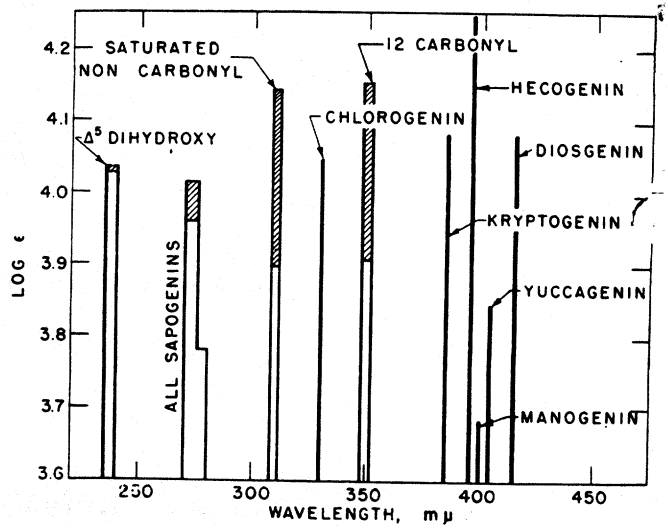


Figure 2. Graph of Log ε *vs.* Wave Length of Absorption Maxima

Solid columns indicate single sapogenins; hollow columns indicate groups of sapogenins. The range of log ε is indicated by the cross hatching; the range of wave length is roughly indicated by the width of the column

material is removed by filtration, and the filtrate is placed on the column. The residue is again treated with hot benzene, cooled, and filtered, and the filtrate is placed on the column. The treatment of the residue is repeated until all of the sample is dissolved and has been placed on the column. The elution with benzene is continued until no more sapogenin is removed from the column with this eluent.

The next eluent, 5% chloroform in benzene, is then used and the elution continued. The concentration of the chloroform is increased to 20%, and finally 20% ethyl alcohol in benzene is used. The benzene and the 5% chloroform in benzene eluents remove the monohydroxy sapogenins from the column, leaving the dihydroxysapogenins adsorbed. The 20% chloroform in benzene removes most of the dihydroxysapogenins, and the 20% ethanol in benzene removes any remaining dihydroxysapogenins.

The procedure with activated alumina differs only in the choice of eluents. The sample is dissolved in 5% chloroform in benzene, eluting with this solvent and then with 20% chloroform in benzene in order to remove the monohydroxysapogenins from the column. Chloroform is then used to remove the dihydroxysapogenins, followed by 20% ethyl alcohol in benzene.

The volume of each eluent that is to be used is determined by the size of the column and by the sample. The point at which the next higher eluent is to be used can be determined by collecting 10 to 20 drops of the eluent on a watch glass and evaporating the solvent to dryness. If a residue remains, the same eluent is continued; if there is no residue, the next higher eluent can be used. Each eluent is collected in a separate container, or containers, and the solvent is evaporated to dryness. Each fraction can then be treated as a single component or as a binary mixture as discussed previously.

DISCUSSION

Chromatography. One of the primary requirements of the method is that the samples to be used must be purified. Any plant residues or resins remaining in the sample would react with the sulfuric acid and render any measurements of the absorbance invalid. Most of the interfering substances are removed in the preliminary processing of the sample (*2*). The remaining interfering substances which are tenaciously held by Florisil can be removed by an adsorption chromatography using this adsorbing agent. At the same time, the sample can be separated into two or more fractions, each of which is a single component or a binary mixture. Several synthetic mixtures, simulating naturally occurring mixtures, were prepared and chromatographed on Florisil with quantitative recovery of the sapogenins.

Factors Affecting the Sulfuric Acid Chromogen. In an effort to standardize the reaction conditions, the effect of the concentration of the sulfuric acid and of the length of heating w.

studied. As commercial sulfuric acid varies in concentration from 95 to 98% acid, it was decided to work with an acid concentration of 94% or lower in order to obtain reproducibility. Using manogenin as a typical example, samples in 85 and 94% sulfuric acid (by volume) were immersed for 1 hour in a water bath at 40° C. The absorptivities were obtained and, as shown in Table III, the values for the 85% sulfuric acid were much lower than the comparable 94% series. Moreover, all these absorptivities were not constant after the 1-hour heating period but increased with time.

Table III. Variation of Absorptivity with Acid Concentration and Length of Heating Period

Wave Length, Mμ	Absorptivity, 85%		Absorptivity, 94%	
	1 hour	16 hours	1 hour	16 hours
250	1.9	12.0	4.9	16.0
275	2.2	17.3	5.6	24.1
295	2.3	17.2	5.2	21.9
310	1.7	13.6	4.5	17.6
330	1.0	10.4	5.4	17.1
350	1.0	14.4	7.0	22.5
396	0.40	13.0	1.5	11.6

Table IV. Comparison of Absorptivities Determined from Pure Sapogenin and Calculated from Binary Mixtures

Wave Length, Mμ	Hecogenin		Tigogenin		Manogenin		Gitogenin	
	Pure	Calcd.	Pure	Calcd.	Pure	Calcd.	Pure	Calcd.
250	16.0	16.4	16.3	15.8	16.0	15.9	17.6	18.3
275	25.6	25.5	24.0	22.1	23.9	23.6	27.4	27.7
295	20.9	21.1	19.3	17.7	21.6	21.1	27.5	27.9
310	19.2	20.1	21.0	20.5	17.8	17.7	29.6	30.1
330	23.3	23.8	19.1	19.9	17.9	18.2	14.0	14.2
350	30.8	29.6	14.1	14.3	23.3	24.1	9.0	9.1

Heating overnight for a total period of 16 hours resulted in stable, reproducible values which, as shown in Table III, were much higher than comparable results obtained after 1 hour. This was particularly desirable because the sensitivity of the procedure was increased at least fourfold. Similar results were obtained with the other common sapogenins.

Determination of Absorptivities. The absorptivities of several sapogenins were determined by two different methods, the results of which appear in Table IV. In the first method, samples of the individual sapogenins were treated with sulfuric acid and the absorptivities were determined. For the second method, binary mixtures of varying percentage composition, composed of hecogenin and tigogenin and of manogenin and gitogenin were treated with sulfuric acid. The absorbance of each solution was measured at the various wave lengths, and the absorptivities of the sapogenins were calculated by means of simultaneous equations.

On the whole, the values found for the single components and those calculated for the binary mixtures are in good agreement. These values also agree with the absorptivities determined from the complete spectral curves obtained from the Cary recording spectrophotometer. One exception, tigogenin, shows nominal discrepancies of 8% at 275 and 295 mμ.

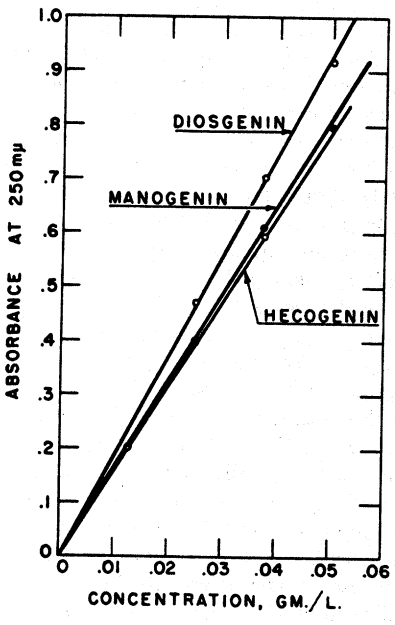


Figure 3. Absorbance of Sulfuric Acid Chromogen as a Function of Concentration of Sapogenin

Measured at 250 mμ

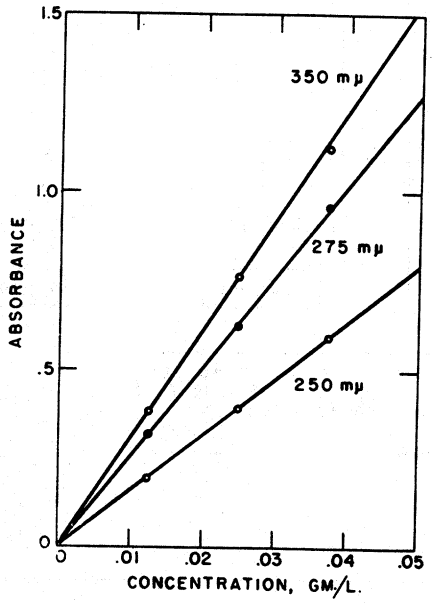


Figure 4. Absorbance of Sulfuric Acid Chromogen *vs.* Concentration of Hecogenin

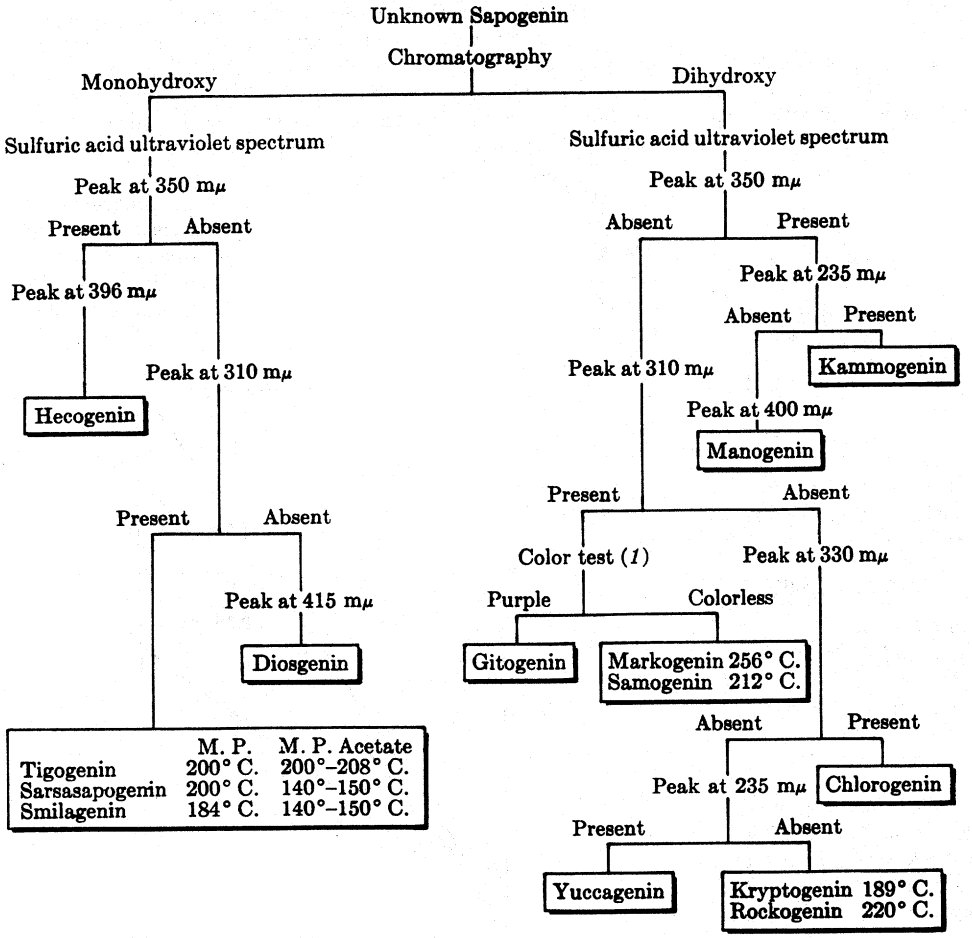
Measurements at three wave lengths

Determination of Adherence to Beer's Law. In order to utilize the method for the estimation of steroidal sapogenins, it was necessary to determine whether the sulfuric acid chromogen follows Beer's law. From complete spectral curves, obtained with a Cary recording spectrophotometer, several wave lengths corresponding to the maxima and minima of some of the sapogenins were selected. Concentrations of 0.0125 to 0.050 gram per liter were prepared, the sulfuric acid chromogen was formed, and the absorbance was measured at the selected wave lengths, using a Beckman DU spectrophotometer with 1.0-cm. quartz cells. Figures 3 and 4 show that the chromogen follows Beer's

Table V. Estimation of Individual and Total Steroidal Sapogenins in Binary Mixtures

Sapogenin Present, Mg.			Sapogenin Found, Mg.		
Hecogenin	Tigogenin	Total	Hecogenin	Tigogenin	Total
1.0	2.0	3.0	1.1	1.8	2.9
2.0	1.0	3.0	2.1	0.8	2.9
1.5	1.5	3.0	1.6	1.4	3.0
2.0	0.5	2.5	2.1	0.2	2.3
0.5	2.0	2.5	0.6	1.8	2.4
Manogenin	Gitogenin	Total	Manogenin	Gitogenin	Total
0.625	1.875	2.5	0.65	1.91	2.56
1.250	1.250	2.5	1.26	1.29	2.55
1.875	0.625	2.5	2.00	0.51	2.51

Table VI. Qualitative Analysis Scheme for Identification of Commonly Occurring Sapogenins



law in the concentration range used. Similar data have been obtained with other sapogenins.

ACCURACY AND PRECISION

The quantity of steroidal sapogenin in a one-component sample can be determined from the absorbance at 250 mμ. Although this is not a maximum, the most reproducible values were found at this wave length. The reproducibility was within ±2% and the average recovery with a number of samples was within ±4%.

For binary mixtures, determinations were conducted at 250 and 350 mμ, where best recoveries were obtained. Various mixtures of hecogenin and tigogenin and of manogenin and gitogenin were prepared. These two mixtures were selected because of their frequent occurrence together in actual practice. The amount of each sapogenin present in the mixture was calculated by means of simultaneous equations, using the appropriate absorptivities (Table II). The results are shown in Table V.

From the data, it is apparent that the total sapogenin content of binary mixtures can be determined. The accuracy in the determination of individual sapogenins is not high, although it is evident that relatively good estimates can be obtained. The amount of the major component can be found with reasonably good accuracy, whereas the error in the minor constituent is large. The precision of duplicate measurements on binary mixtures is within ±2%.

IDENTIFICATION OF STEROIDAL SAPOGENINS

Complete spectral curves of the steroidal sapogenins available and of some of the derivatives of these compounds were obtained in the region of 220 to 600 mμ with a Cary recording spectrophotometer. The maxima of these curves, as well as log ε, are shown in Table I. All of the sapogenins show a maximum in the region 270 to 275 mμ. This is probably due to the spiroketal structure of the *E* and *F* rings or to its open chain derivative, since the 3-desoxy and the 3-keto sapogenins also have peaks in this region, as does kryptogenin, which has the *E* and *F* rings open. Cholesterol, a non-sapogenin, which also gives a chromogen upon treatment with sulfuric acid, does not have a peak in this region.

A peak at 350 mμ occurs only with the 12-keto sapogenins, as shown by the fact that nonketonic and 3-keto sapogenins do not have maxima at this wave length, nor does kryptogenin, a 16,19-diketo compound. The 2,3-dihydroxysapogenins with a 5–6 double bond have a characteristic peak near 235 mμ, which is lacking in the spectrum of a 3-hydroxy, 5–6 double bond compound such as diosgenin. However, the latter compound has characteristic absorption maxima in the region 400 to 600 mμ. The absorption maxima of the latter type compound are probably due to dehydration of the 3-hydroxyl, followed by the production of a complex conjugated system. For example, 3-desoxydiosgenin, which does not have a 3-hydroxyl group, has a spectrum that lacks absorption in the 400 to 600 mμ region and resembles that of the saturated compounds.

The spectra of the saturated, noncarbonyl sapogenins, whether monohydroxy, dihydroxy, or desoxy, are essentially similar, exhibiting a typical absorption maximum near 310 mμ. An exception to this is chlorogenin, which has a characteristic peak at 330 mμ. This peak may be due to the effect of 6-α-hydroxyl, which occurs only in chlorogenin. Many sapogenins have characteristic peaks in the region 375 to 600 mμ which can be useful in identifying them. The data are summarized in Figure 2.

From the foregoing discussion, it is apparent that many of the steroidal sapogenins can be characterized by the ultraviolet spectra of the sulfuric acid chromogens. However, a number of sapogenins, particularly the saturated noncarbonyl compounds, have spectra which are too similar to permit conclusive identification. If one uses chromatographic adsorption to distinguish merely between the easily eluted monohydroxy sapogenins and the more tenaciously adsorbed dihydroxysapogenins, and, in certain cases, utilizes Kofler micro melting points, a complete identification of at least 13 of the commonly encountered sapogenins can be made with as little as 5 to 10 mg. A qualitative scheme is given in Table VI using these three criteria: chromatographic behavior, spectrum of the sulfuric acid chromogen and melting point.

ACKNOWLEDGMENT

The authors appreciate the assistance of Mary-Anne Morris in obtaining the complete spectral curves of the sulfuric acid chromogens

LITERATURE CITED

(1) Diaz, G., Zaffaroni, A., Rosenkranz, G., and Djerassi C., *J. Org. Chem.*, 17 747 (1952)

(2) Wall, M. E., Krider, M. M., Rothman, E. S., and Eddy, C. R., *J. Biol. Chem.*, 198, 533 (1952).